ACTIVITIES OF DIRECTLY AND INDIRECTLY ACETYLATED CHYMOTRYPSINS*

By A. K. Balls and C. A. Ryan

WESTERN REGIONAL RESEARCH LABORATORY, ALBANY, CALIFORNIA†

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The introduction of one acetyl group into the chymotrypsin molecule by reaction with p-nitrophenyl acetate gives rise to an enzymatically inactive monoacetylated protein that rapidly loses its acetyl group and becomes active again in solutions of dilute alkali or primary alcohols.[1, 2] On the other hand, the well-known polyacetylation of proteins with acetic anhydride, as described by Fraenkel-Conrat, Bean, and Lineweaver,[3] yields active (or potentially active) products with chymotrypsin and chymotrypsinogen in which much of the acetyl remains rather firmly bound. The following paper is meant to give a first account of some observations made on polyacetylated chymotrypsin[3a] and chymotrypsinogen that may be of interest because they indicate that these artifacts behave enzymatically on several counts in very different fashion from their naturally occurring counterparts.

Marked differences between the behavior of derived chymotrypsins and the naturally occurring enzyme were observed by Jansen *et al.*[4] who found the pH optimum of esterolysis[4a] to be altered by acetylation. They also observed that esterolytic activity is much less reduced by oxidation with periodate than is proteolytic activity. The chemical modifications made by Vallee *et al.*[5] on carboxypeptidase A have led to even more striking changes in the properties of that enzyme. The peptolytic activity may be caused to disappear altogether, while at the same time the esterolytic activity increases manyfold.

Multiple acetylation of chymotrypsin led in our hands to a product of mediocre activity, compared to the original enzyme, on both TEE and protein. However, when a solution was maintained at neutrality for some time, its esterase activity approximately doubled and thus became about equal to that of the natural enzyme. The proteolytic activity remained unchanged.

Observations on chemically modified chymotrypsinogens, including acetylated chymotrypsinogen, have been reported by Wilcox in a series of papers.[6–9] They leave no doubt that in many instances such modified proteins yield active ester-splitting enzymes on treatment with trypsin. In the case of the acetylated zymogen, the observed esterolytic activity was as high as that obtained from natural chymotrypsinogen under like conditions.[7]

Since some of the groups in active chymotrypsin, including the so-called "active center" itself, are evidently not available for reaction in chymotrypsinogen, we thought it might be of interest to compare the active protein obtained by the direct acetylation of chymotrypsin with that obtained by activation of the previously acetylated zymogen. It was found that acetylated chymotrypsinogen differed from the naturally occurring protein in its behavior with trypsin. Unlike the natural protein, it formed little or no active enzyme with trypsin at pH 7.0-7.2, unless an electrolyte was also present in appreciable concentration or, alternatively, some natural (i.e., not acetylated) chymotrypsin. Moreover, the product of activation differed from both natural and directly acetylated chymotrypsins in that its estero-

lytic activity (measured with TEE) was about twice that observed with the natural protein, although proteolytic activity remained at the same low level as that of the directly acetylated enzyme.

Materials and Methods.—Chymotrypsin was prepared from 8 times recrystallized chymotrypsinogen as described by Northrop, Kunitz, and Herriott.[10] Both proteins were acetylated at 0° in half-saturated sodium acetate solution with 1.3 times their weight of acetic anhydride added over one hr. Thereafter, the material was dialyzed and finally dried *in vacuo* while frozen. Titration of the resulting products with NaOH in the presence of formaldehyde (formol titrations) showed that acetylation had caused a loss of about 80% of the titratable groups of the chymotrypsinogen and 71% of those in the chymotrypsin used. The molecular weights of these derivatives, as well as of the original proteins, have all been taken at the approximate value of 25,000.

L-tyrosine ethyl ester (TEE) was recrystallized from commercial material and checked with respect to optical activity. Other reagents were good grade commercial products.

The esterolytic activity of chymotrypsin was determined by the initial rate of hydrolysis of 20 μmoles of L-tyrosine ethyl ester in 2.0 ml of 0.025 M $CaCl_2$, pH 6.25 at 25° as measured by essentially continuous titration with 0.10 N NaOH from a microburette. The same apparatus was used for the titration of the acetylated proteins in μ-molar quantities. The milk-clotting capacity[11] of the enzyme was determined at 40° on 10 ml portions of a 10% solution of dried skim milk, pH 6.2–6.3, made 0.01 M with $CaCl_2$ shortly before use. Under these conditions, both milk-clotting and esterolytic activities are for some time linear functions of the quantity of enzyme employed. Specific activity (Sp. Act.) is defined as moles of ester split per minute per gm of protein. Milk-clotting activity, likewise a constant for a given preparation, is expressed as $K = 1/(t)\,(mg)$, where t is clotting-time in minutes and mg, the milligrams of enzyme present. The digestion of casein is reported as the increase in optical density at 280 mμ obtained with 100 μg of enzyme protein by the procedure of Kunitz,[12] except that the reaction time was 15 min at 25°.

The action of hydroxylamine was measured by the method of Hestrin[13] as slightly modified for use with proteins.[14]

Activation of Acetylated Chymotrypsinogen.—Trypsin was added to the slightly turbid solution of the acetylated zymogen in water, followed by dilute NaOH until the pH remained constant at 7.0.[14a] Aliquots of such a solution were then mixed with solutions of the additions to be tested, also at pH 7.0, in appropriate concentrations. Experiments carried out at 5° contained one part of trypsin protein per thousand of zymogen; those at room temperature (25°) were made with one part of trypsin to 200 of zymogen. The former series required several days to reach full activity in some cases; the latter, only a few hours. No differences in principle were seen between the two series of experiments. It was observed that all salts were not equally efficient promoters of activation. Maleate and citrate buffers, 0.05 M, were particularly satisfactory, whereas 0.3 M NaCl and 0.5 M Na acetate did not produce as rapid activation. Table 1 gives some typical examples. The substitution of natural chymotrypsin for any electrolyte is noteworthy, as is the failure of acetylated chymotrypsin to act in a similar fashion.

The esterolytic activity developed from the acetylated zymogen usually amounted to 150–200 per cent of the value found for the natural enzyme or for the directly acetylated enzyme after neutralization. It was not accompanied by an increase in milk-clotting (or casein-digesting) power. The rate at which these two functions of the enzyme developed during the activation process is of interest because the natural and the acetylated chymotrypsinogens differed in this respect, as Table 2 shows.

An inspection reveals that this difference in behavior between natural and acetylated zymogens is not owing to the faster development of milk-clotting power in the

TABLE 1

TRYPTIC ACTIVATION OF ACETYLATED CHYMOTRYPSINOGEN

Addition	Temp.	Specific Activity (TEE)* Hr 0.5	1.0	1.5	20	40	66
Water only	5				0	0	0
$CaCl_2$, 0.025 *M*	5				0.014	0.071	0.069
Maleate, 0.10 *M*	5				0.050	0.071	0.079
NaCl, 0.30 *M*	5				0.033	0.063	
Na acetate, 0.50 *M*	5				0.041	0.062	
Water only	25	0.006	0.008	0.009			
$(NH_4)_2SO_4$, 0.05 *M*	25	0.034	0.055	0.057			
Maleate, 0.05 *M*	25	0.038	0.057	0.069			
Natural chymotrypsin† (Sp. Act. 0.040)	25	0.058	0.058	0.058			
Directly acetylated chymotrypsin†	25			0.003			
The activation product of acetylated chymotrypsinogen†,‡	25			0.007			

* Based on weight of the zymogen used for activation.
† Ten % of weight of zymogen. Correction has been made for the additional activity thus introduced.
‡ Made by rapid activation in maleate buffer as shown above, then dialyzed.

acetylated case, thus keeping pace with the greater esterolytic activity formed, for the reverse is true; the esterolytic activity developed more slowly, although eventually it went further. Only at the very beginning was there any resemblance between the two systems.

TABLE 2

RATE OF TRYPTIC ACTIVATION OF NATURAL AND OF ACETYLATED CHYMOTRYPSINOGEN

Time, min	Activity Developed* Chymotrypsinogen Milk K	TEE Sp. Act.	Ratio TEE/Milk × 10^2	Acetylated Chymotrypsinogen Milk K	TEE Sp. Act.	Ratio TEE/Milk × 10^2
5	1.4	0.035	2.5	0.6	0.018	3.0
10	2.4	0.049	2.0	1.0	0.030	3.0
30	2.8	0.044	1.6	2.2	0.062	2.8
60	2.6	0.040	1.5	2.4	0.070	2.9
90	2.4	0.040	1.7	2.4	0.070	2.9

* Based on weight of zymogen.

There is no need to invoke the existence of two different sites in the protein in explanation of these results. The behavior of the natural zymogen is consistent with the formation of a series of intermediates such as what is known to form under these conditions.[15] By the same token the acetylated zymogen seems not to undergo the same type of progressive degradation. This could account for its high (and stable) esterolytic activity, but not for the observed low proteolytic power. However, it is evident from the observation of Antony[16] that proteolytic activity in heavily acetylated chymotrypsin may be influenced by groups stable at pH 8, but labile with hydroxylamine (obviously other than the group(s) in question here).

Directly Acetylated Chymotrypsin.—As prepared by us, the protein formed an opalescent solution in water, pH 4.5–4.7. About 4–5 equivalents of NaOH per mole of protein were required to bring the solution to neutrality. Thereafter, a slow liberation of acid took place, lasting about an hour and amounting to one molar equivalent. The specific esterolytic activity was approximately doubled by this treatment, thus reaching about the same level as that of the natural enzyme, but

still only half or two-thirds the activity shown by the product of activation of acetylated chymotrypsinogen. The same result was obtained by storing a solution of the protein in neutral maleate buffer overnight at 5°. Table 3 gives comparable values for esterolysis, milk-clotting, and also casein digestion. The milk-clotting power of the directly acetylated enzyme did not increase on neutralization. The increase in ester-splitting activity is permanent. It is not reversed by reacidification.

TABLE 3

Esterolytic and Proteolytic Activities of Acetylated Proteins

	TEE Sp. Act.*	Milk K*	Casein digestion*
α-Chymotrypsin	0.040	3.1	1.11
Directly acetylated chymotrypsin, pH 4.6	0.018	1.7	—
Directly acetylated chymotrypsin, 20 hr in .05 *M* maleate, pH 7.0	0.040	1.8	0.78
Activation product of acetylated zymogen in .05 *M* maleate, pH 7.0	0.064	2.6	0.78

* See text under *Methods*.

Action of p-Nitrophenylacetate and Hydroxylamine on Acetylated Chymotrypsins.—The behavior of directly acetylated chymotrypsin strongly suggests that the active site of the enzyme has remained partly acetylated throughout the later steps (dialysis and lyophylization) in the preparation of the protein. Ordinary monoacetyl chymotrypsin is unstable under such conditions, but it may be postulated that the presence of other acetyl groups in the protein slows down the decomposition of this particular group through something akin to steric hindrance. To test this hypothesis, the protein was treated with p-nitrophenylacetate under conditions previously described.[14] The burst of free nitrophenol that takes place when the protein and this reagent are mixed may be taken as a measure of the "active" group that is available for acetylation. A sample (25 mg) of directly acetylated chymotrypsin was dissolved in 0.10 *M* PO_4 buffer, pH 6.2, and tested in this way when the solution was 10 min old, and also after 20 hr at 5°. Table 4 (Items 5, 6, and 7) shows that a marked increase in the burst of nitrophenol accompanied the increase in esterolytic activity.

The presence of an active acetyl group was also indicated by the reaction with

TABLE 4

Action of p-Nitrophenylacetate and Hydroxylamine on Acetylated Chymotrypsins

	Hydroxamic acid* μmoles/25 mg pH 6.0	Hydroxamic acid* μmoles/25 mg pH 11.2	Burst of p-NO_2 phenol	Sp. Act. TEE
(1) Acetylated chymotrypsinogen	0.8	2.9	—	—
(2) Same, after activation‡	0.3	1.3	0.90	0.078†
(3) Same, activated‡ after treatment with 2 *M* hydroxylamine at pH 6				0.073†
(4) Same,‡ except hydroxylamine at pH 11.2				0.065†
(5) Directly acetylated chymotrypsin (pH of solution, 4.6)	1.2	2.2	0.52	0.017
(6) Same, after 20 hr, 5°, at pH 7.0	0.1	1.1	0.82	0.030
(7) α-Chymotrypsin	0.2	0.2	0.80	0.040
(8) α-Chymotrypsinogen	0.2	0.2	—	—

* After subtraction of the "blanks" (0.2) shown below for the unacetylated proteins (items 7 and 8).
† Based on weight of zymogen used.
‡ In 0.05 *M* maleate, pH 7.0, at 25° for 2 hr.

hydroxylamine. Table 4 shows the amount of hydroxamic acid formed at pH 6.0 and 11.2 by several typical acetylated preparations.

The presence in the acetylated zymogen of so much active acetyl is surprising, but it has been observed in three separate preparations. However, this appears to have no connection with the increase of esterolytic activity reported here; for although the *removal* (at pH 7) of one O-acetyl (or imidazole-acetyl) practically doubled the esterolytic activity of the directly acetylated protein, treatment of the acetylated zymogen with hydroxylamine at either pH 6 or pH 11.2 had no effect on the esterolytic activity of the activation product. Therefore, the acetyl group responsible for this increased activity is not apt to be attached to hydroxyl in tyrosine or to imidazole[17] but is in all probability an N-acetyl configuration. The effect of such a substituent might well be a negative one. Instead of increasing the activity, it could simply prevent a loss thereof. One is inevitably reminded of the breakdown of the π-chymotrypsin of Jacobsen[18] to proteins of lower proteolytic activity, and wonders whether an appropriate substituent group in the zymogen might not hinder that change.

The Role of Chymotrypsin in the Activation of Acetylated Chymotrypsinogen.—The process of activation, which always involved trypsin (soy-bean trypsin inhibitor prevented it), depended also on the action of electrolyte such as sodium chloride or, alternatively, on the action of natural chymotrypsin. So far as present observations have gone, the end result appears to be the same, but this can hardly be the case. Both acetylated zymogen and directly acetylated chymotrypsin rapidly liberated acid groups titratable at pH 7.0 in 0.5 M NaCl. But we found that other proteins, for example, bovine serum albumin, acted the same way. Such changes may bespeak an alteration in the tertiary structure of the protein.[19] Chymotrypsin, however, liberates no acid rapidly from either acetylated protein. Its action could be expected rather on the primary structure. This viewpoint is borne out by the observation that chymotrypsin attacked the acetylated zymogen and rendered it activable by trypsin in the absence of electrolyte. This was shown by treatment of acetylated chymotrypsinogen with chymotrypsin at pH 7.0 in the absence of trypsin and salts. A tenfold excess of diisopropyl fluorophosphate (over the chymotrypsin) was then added. A test 30 min later with tyrosine ester showed that all chymotryptic activity was gone. Thereafter, the addition of trypsin alone to the isolated zymogen produced a specific activity of 0.067 in 2 hr at 25°.

Summary.—The introduction of a number of acetyl groups into α-chymotrypsin by treatment with acetic anhydride reduced both proteolytic and esterolytic activities. The latter activity, but not the former, was restored, usually to about the level found for the unacetylated enzyme, by standing in neutral or slightly alkaline solution. The protein lost one active acetyl group during this treatment, thus acting like monoacetyl chymotrypsin prepared with nitrophenylacetate.

Acetylated chymotrypsinogen could be activated by trypsin, but only under special conditions that are not necessary for the naturally occurring protein. The presence of an electrolyte or else the addition of some natural (i.e., unacetylated) chymotrypsin was required. When either of these conditions was fulfilled, the end product of activation showed about the same proteolytic activity as directly acetylated chymotrypsin, but the esterolytic activity was nearly double that found for either the directly acetylated or the natural enzyme. The effect of natural

chymotrypsin in this activation was at least in part on the acetylated chymotrypsinogen itself before the attack thereon by trypsin. The failure of hydroxylamine to change the potential activity of the zymogen indicated that the markedly increased esterase activity of the activation product is owing to the presence of an N-acetyl derivative. A simple viewpoint that fits the presently known facts is that such an N-acetyl group is so situated as to offer "steric hindrance" to the rearrangement or further degradation of the enzyme to a less active form.

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[3a] The terms "chymotrypsin" and "chymotrypsinogen" refer here, unless otherwise stated, to the usual α-forms of these proteins.

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[10] Northrop, J. H., M. Kunitz, and R. M. Herriott, *Crystalline Enzymes* (New York, 1948), p. 262.

[11] We have in general regarded the outstanding milk-clotting activity of chymotrypsin as a measure of its proteolytic activity. The digestion of casein[12] has also been used here in some instances as an additional criterion, but it should be remembered that all such methods largely depict peptolytic rather than proteolytic activity. The measurement of actual protein-splitting, particularly in the early stages, has not as yet been achieved with satisfactory accuracy except (perhaps) by the measurement of viscosity. Gelatin is already too greatly modified to serve as a typical protein. Casein with added calcium salts is, however, an excellent substrate of chymotrypsin.

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[14a] It should be noted that the addition of *ca.* 4 equivalents of NaOH is required to neutralize a solution of the acetylated zymogen in water, and much more is needed to maintain an alkaline pH for any length of time. Activation has been observed to occur at *ca.* pH 9 without other added electrolyte; but whether this is owing to the high pH, to the electrolyte already present, or to both, is not clear. Further dialysis of an alkaline solution against water, or passage through cephadex, brings the pH level back to *ca.* 5.6 and also restores the need of added electrolyte for activation at pH 7.0.

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